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Acute Treatment With Herbal Extracts Provides Neuroprotective Benefits in In Vitro and In Vivo Stroke Models, Characterized by Reduced Ischemic Cell Death and Maintenance of Motor and Neurological Functions

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The present study explored the prophylactic and restorative benefits of cacao and red sage using both in vitro and in vivo models of stroke. For the in vitro study, we initially exposed primary rat cells to the established oxygen-glucose deprivation (OGD) stroke model followed by reperfusion under normoxic conditions, then added different cacao and sage concentrations to the cell culture media. Trypan blue cell viability results revealed specific cacao and sage dosages exerted significant therapeutic effects against OGD-induced cell death compared to cultured cells treated with extract vehicle. We next embarked on testing the therapeutic effects of cacao and sage in an in vivo model of stroke when extract treatment commenced either prior to or after transient middle cerebral artery occlusion (MCAo). Significant reduction in ischemic cell death within the peri-infarct area coupled with better performance in routine motor and neurological tasks were demonstrated by stroke animals that received cacao or sage extracts prior to MCAo compared to those that received the extracts or vehicle after MCAo. In summary, the present results demonstrate that neuroprotective effects were afforded by plant extract treatment, and that the in vitro stroke paradigm approximates in vivo efficacy when considering prophylactic treatment for stroke.

Key words: Oxygen-glucose deprivation (OGD); Stroke; Prophylactic; Middle cerebral artery occlusion (MCAo); Plant extracts

INTRODUCTION

Stroke is the third leading cause of death in the Western world, and there is limited successful treatment (19). The only drug currently approved by the U.S. Food and Drug Administration, tissue plasminogen activator, has to be administered within 3 h after the onset of stroke in order to be effective (17). This lack of an effective treatment beyond the acute phase of stroke prompted us to investigate the therapeutic benefits of preemptive use of dietary supplements to help reduce the severity of the stroke. Along the lines of enhancing host neurogenesis by dietary supplementation [see, e.g., (1,27)], we now focus on specific compounds of these diets in order to reveal the most effective active ingredients as we translate these treatments into clinical applications. The overarching notion we advanced in these articles was that dietary supplementation via its active components largely afford neuroprotection in stroke via a neurogenesis-based mechanism.

Some dietary supplements have been shown to exert varying degrees of benefit against the detrimental effects of stroke in animal models when given prophylatically. For instance, Wang et al. (23,24) have demonstrated re-

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duced ischemia-induced cell death following dietary grape supplementation, whereas Wang et al. (25) demonstrate that blueberries, spinach, and spirulina supplementation of the diet all exert some benefit towards infarct size and behavioral deficits following a transient middle cerebral artery occlusion (MCAo). The recent study by Yasuhura et al. (27) also demonstrated some functional recovery and neurological protection following dietary supplementation with a proprietary formulation known as NT-020.

The mechanism of action of these supplements is unclear, but may relate to free radical scavenging, and other antioxidant effects, anti-inflammatory effects (25), and neurogenesis (27). Additional dietary studies with curcumin suggest that an additional mode of action could be reduced blood–brain barrier damage (10).

Cacao contains high levels of potentially advantageous compounds including flavanols (16), and some epidemiological evidence suggests that high flavanol intake could confer some degree of protection against common diseases such as ischemic heart disease, cancer, and stroke, among other debilitating disorders (2), possibly by a peripheral vasodilator response and increased cerebral perfusion (8). Animal models suggest that long-term administration of the cacao polyphenol extract acticoa may also provide neuroprotection against the age-induced loss of neurons (4).

Red sage (*Salvia miltiorrhiza*, also known as "danshen" in China) is also a mixture of different compounds (9) and has been used as a traditional Chinese medicine for the treatment of many disorders including coronary heart diseases, renal diseases, and inflammation for many years (13,14). It has also been shown to possess antioxidant properties against peroxynitrate-related disorders (11) and differentiate human umbilical cord-derived and bone marrow-derived mesenchymal stem cells into neuronal-like cells (14,15,26). A recent study has shown therapeutic benefits of different extracts of red sage against animal models of stroke, possibly by reducing cerebral energy metabolism and acting as an antioxidant (22).

In this pilot study we examined the effects of extracts of dietary supplements such as red sage and cacao in an in vitro cell culture model of stroke [oxygen-glucose deprivation (OGD)], as well as an in vivo animal model of stroke (MCAo).

MATERIALS AND METHODS

Coded and proprietary extracts of red sage and cacao were provided by Herbal Science (Naples, FL). The extracts were stored at 47°C until solubilization in 1% dimethyl sulfoxide (DMSO) in Tris-HCl, pH 7.4, by 3–5 1-s sonications (Branson Sonifier 250; Branson, Danbury, CT) to generate 100× stocks, which were sterile filtered and then further diluted with DMEM/10% FBS to give high (2000 ng/ml) or low (200 ng/ml) concentration solutions for the in vitro OGD studies. The 100× stock solutions were prepared fresh each day. Unless specified otherwise, chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

The OGD studies were performed using rat primary neurons (BrainBits, Springfield, IL) isolated from E18 rats and plated at 4×10^4 cells/well in poly-L-lysine-coated plates and cultured in 200 μl Neural Medium (NbActive 4; BrainBits) for 4 days. On the 5th day the media was replaced with OGD media (116 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 1 mM NaH_2PO_4, 26.2 mM $NaHCO_3$, 0.01 mM glycine, 1.8 mM $CaCl_2$, pH 7.4), placed in a humidified chamber (Plas Labs, Lansing, MI), and exposed to 95% N_2 and 5% O_2 at 37°C for 15 min before the chamber was sealed and the cells exposed to the hypoxic conditions for 90 min. The cells were then placed under reperfusion (normoxic) conditions for 2 h (air and 5% CO_2 at 37°C with 5 mM glucose added to the media). Half the media was then replaced with media containing the low or high doses of plant extracts (to give final concentrations of 100 and 1000 ng/ml, respectively) or vehicle (1% DMSO). Vehicle wells were present on each plate to act as controls for each group ($n = 3–10$). After 3 h, 0.4% trypan blue (Invitrogen, CA) was added to the cells and the trypan blue exclusion method was conducted to obtain mean viable cell counts based on three randomly selected areas (0.2 mm^2) in each well.

Based on the results from the OGD study, one optimal extract of cacao (HS1375) and red sage (HS725) was chosen for in vivo studies in which Sprague-Dawley rats were treated with the extracts by oral gavage either before or after transient MCAo. The doses used were extrapolated to equivalent high (270 mg/kg) and low (27 mg/kg) concentrations (20) and were prepared as before except they were diluted with water instead of DMEM and the solutions were more concentrated. All animal experiments were approved by the University of South Florida IACUC and conformed to NIH guidelines.

The post-MCAo treatment was performed first in line with the in vitro data. The MCAo protocol was performed as previously described (5). Briefly, 35 male Sprague-Dawley rats of approximately 300 g (range 286–328 g, mean 307.4 ± 12) were anesthetized, the MCA was isolated, and an intraluminal suture inserted to temporarily occlude the MCA. Laser Doppler was used to monitor cerebral blood flow during the procedure and confirm occlusion (and reperfusion). After 60 min the intraluminal suture was removed and the rat sewn up and allowed to recover for 3 h before being administered orally 1 ml of either low (27 mg/kg, $n = 14$) or high (270 mg/kg, $n = 14$) dose red sage ($n = 14$)

or cacao ($n = 14$) or vehicle (1% DMSO in distilled water; $n = 7$). Behavioral testing, which consisted of the elevated body swing test, Bederson pawgrasp, Bederson forelimb, and the total Bederson test, was performed as previously described (7) on the following 2 days, and on the 4th day the rats were terminally anesthetized before perfusion with cold saline. The brain was then removed and sliced into 2-mm sections before incubation with cold 2% triphenyl tetrazolium chloride (TTC) for 10 min prior to cold 4% paraformaldehyde fixation. Infarct size was then calculated for each group.

As a pretreatment, 7–8 rats in each group were given low (27 mg/kg) doses of red sage, cacao, or vehicle by oral gavage immediately before MCAo. Behavioral testing and TTC staining were performed as previously described and the infarct size determined.

Statistical Analysis

Cell viability data for each extract was compared by ANOVA and Tukey-Kramer post hoc testing with a value of $p < 0.05$ considered significant.

RESULTS

Post-OGD Treatment With Herbal Extracts Confers Protection

Four red sage extracts (HS725, HS728, HS730, and HS733) and two cacao extracts (HS1375 and HS1380) were tested for their ability to confer neuroprotection after OGD at low and high doses in several separate experiments. The extracts did not appear to show any benefit in the absence of OGD (data not shown), but at least one low dose and high dose extract of red sage and cacao were shown to significantly increase viability compared with the vehicle, as shown by trypan blue exclusion (Table 1).

Post-MCAo Treatment With Herbal Extracts Does Not Confer Protection

No significant differences between the EBST, the Bederson total (or other) behavioral tests, or infarct size were observed with either a low or high dose of either herbal extract compared with the vehicle group when administered after MCAo (Figs. 1 and 2). However, it is worth noting that the variation in the vehicle data for the Bederson's total and infarct size may be masking an effect, as a possible trend towards protection is shown with the red sage extract.

Table 1. The Significant Neuroprotection Observed Against Oxygen-Glucose Deprivation With the Different Extracts of Red Sage (HS725, HS728, HS730, HS733) and Cacao (HS 1375, HS 1380)

Compound	Low Dose	High Dose
HS725	$p < 0.01$ ($n = 4$)	$p < 0.05$ ($n = 10$)
HS728	NS ($n = 6$)	$p < 0.05$ ($n = 6$)
HS730	NS ($n = 6$)	NS ($n = 6$)
HS733	NS ($n = 10$)	$p < 0.05$ ($n = 10$)
HS1375	$p < 0.01$ ($n = 3$)	$p < 0.001$ ($n = 10$)
HS1380	$p < 0.01$ ($n = 4$)	$p < 0.05$ ($n = 4$)

Herbal Extract Pretreatment Confers Protection With Respect to Infarct Size After MCAo

Pretreatment with a low dose of both herbal extracts led to a significant reduction in the EBST test ($p < 0.0001$), the Bederson total test ($p < 0.05$), and the infarct size after MCAo compared with vehicle ($p < 0.0001$) (Figs. 1 and 2).

DISCUSSION

Herbal extracts such as red sage and cacao are a plethora of different compounds and it is unclear whether each ingredient has a role or whether there are specific moieties that provide the most benefit. This pilot study suggests that certain extracts may be potentially useful as a prophylactic treatment for stroke and possibly other neurodegenerative disorders. Our results suggest that there is some benefit from a single dose of either of these extracts given orally immediately before a stroke. The lack of an effect when administered after the stroke suggests that the neuroprotective action occurs early in the progression of the stroke, either working at the time of the stroke or in the reperfusion period directly after the stroke. Because there is evidence for an antioxidant action of both the red sage extracts (11, 22) and the cacao (18), it is certainly plausible that the extracts could work by reducing the production of free radicals brought about by the glutamate excitotoxicity triggered by reperfusion (6). Indeed, a component of red sage—cryptotanshinone—has been shown to inhibit glutamate-induced toxicity in vitro (28). When administered 3 h after reperfusion, this could be too late to have a beneficial effect. However, the in vitro experiments may suggest that this is not entirely the case, because the extracts were effective when given 2 h after reperfusion following OGD. This could reflect differences between the two models or may relate to the pharmacokinetics of the extracts. Studies have shown that some of the components of red sage when given intraperitoneally are capable of crossing the blood–brain barrier to a limited extent and this is significantly enhanced following MCAo (12,30), implying that, in principle, the ability of the compounds to reach the infarct area may not be a determining characteristic with respect to their effects. The bioavailability of the herbal extracts may be variable following oral administration, depending on which specific ingredient is measured (16,29). Accordingly, the

influence of the pharmacokinetics of the extracts on their therapeutic actions will need to be explored in future studies.

In addition, Tang et al. (21) showed reduced impairment of the blood–brain barrier following an intraperitoneal injection of tanshinone, one of the ingredients of red sage. That the herbal extracts employed here also promoted repair of the blood–brain barrier, which may have contributed to the resulting functional benefits, will also warrant further investigations.

A single oral dose of extract appeared to confer some degree of neuroprotection against MCAo, which suggests that they may have a potential role as a prophylactic treatment in humans. A form of cacao is already in the food chain as chocolate and there is some suggestion that at least some of the benefits remain in this food staple, making it a potentially useful means of administering a prophylactic treatment. However, further experiments to confirm that long-term treatment prior to MCAo and toxicity studies are necessary before their prophylactic use could be endorsed.

A combined pre- and posttreatment regimen of each

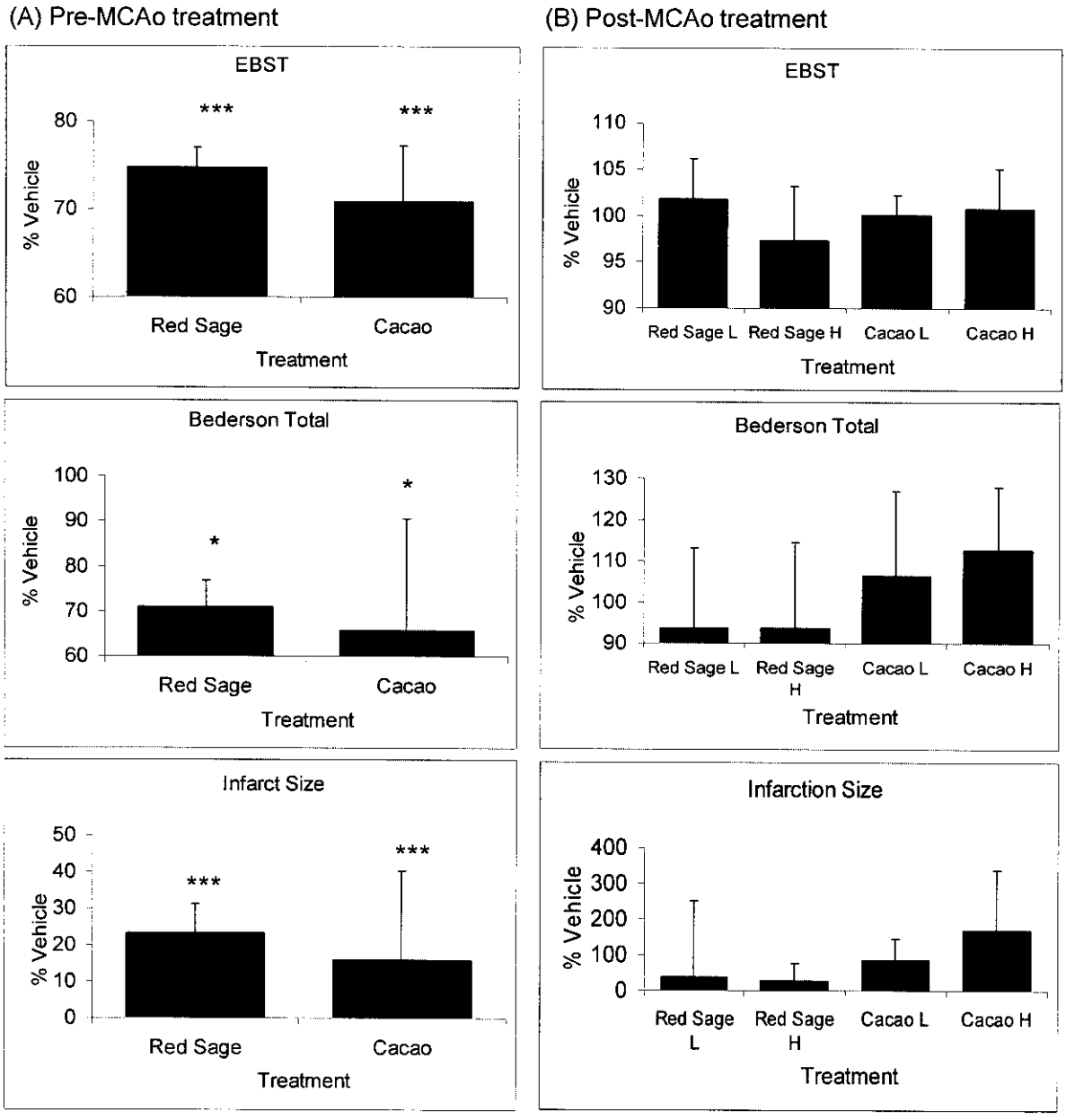

Figure 1. Graphs showing the EBST, Bederson total, and infarct area ratio data following pretreatment of a low dose of either extract (A) or post-MCAo treatment of low or high doses of either extract (B). *$p < 0.05$, ***$p < 0.001$ compared to vehicle.

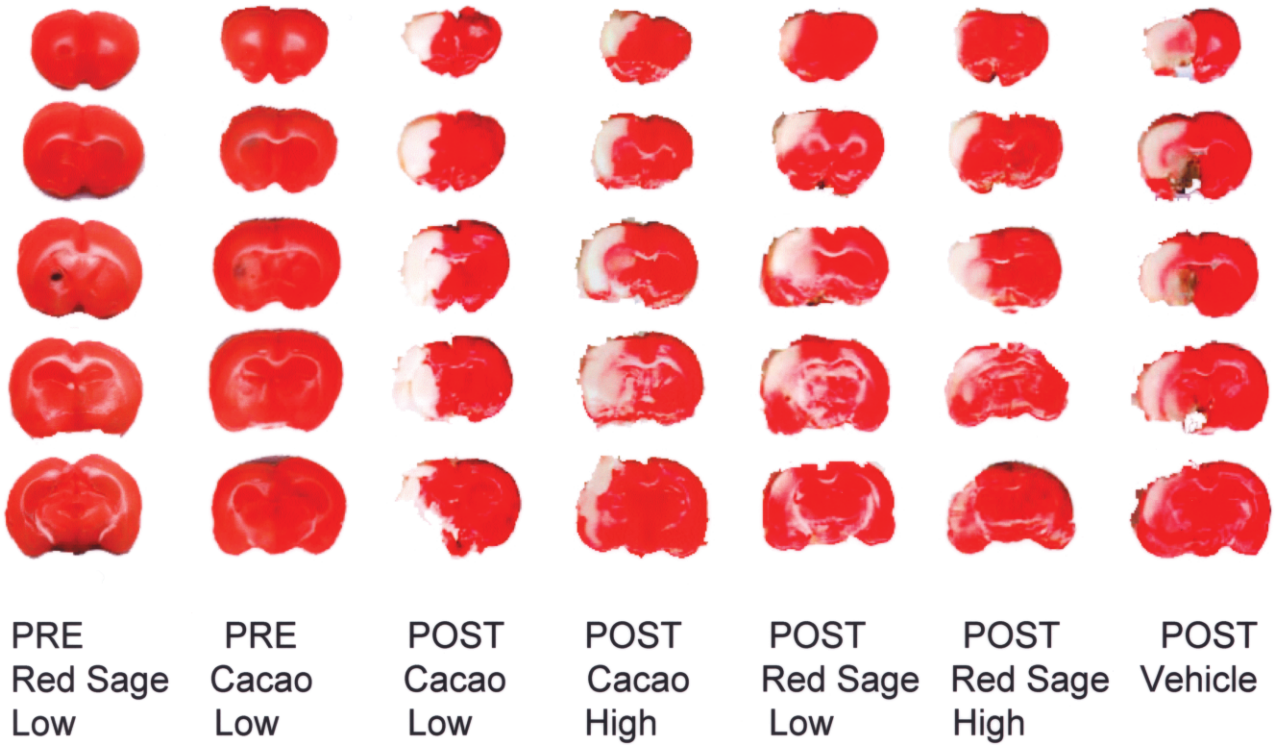

Figure 2. Triphenyl tetrazolium chloride (TTC) staining of brain slices demonstrating infarct size. A typical example of staining for each experimental group is shown except for the pretreatment vehicle. This was similar to the posttreatment vehicle example shown.

extract may also prove to be effective or alternatively use of a cocktail of both extracts (or the complex mixture of compounds found in the raw product or with a mixture of other food supplements) may be the most therapeutic as they exert their benefits via multiple neuroprotective processes, resulting in additive beneficial effects. The synergistic effects of the compounds present in formulation NT-020 would appear to support this possibility because a 20-fold less concentrated combination of the ingredients produced similar or greater benefit than the individual compounds alone (3,20,27). The extracts could also prove to be useful as an adjunct therapy with cell transplantation, by acting to stabilize either the transplanted cell, help bolster their neuroprotective action, or possibly even promote neurogenesis.

Further studies should be geared towards determining the optimal dose and time (and whether chronic administration could be beneficial) as well as determining possible mechanisms of action such as effects on apoptosis, inflammation, or neurogenesis and stem cell proliferation (3,27). If neurogenesis is a viable method of action for these extracts, then it is likely that the generation of new cells could be determined following dosing by the use of BrdU and other markers of stem cell proliferation. This would allow the possibility that the promotion of new cell formation could confer protection against MCAo, although this may depend on the time course of action of the extracts with respect to neurogenesis.

In summary, the present study shows that a single dose of extracts of red sage or cacao provide neuroprotection against OGD when administered after treatment in vitro and neuroprotection and behavioral benefit when administered prophylactically in an animal model of stroke. This raises hope for a potential prophylactic treatment for patients at risk of stroke.

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